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Effects of nesfatin-1 on food intake and LH secretion in prepubertal gilts and genomic association of the porcine *NUCB2* gene with growth traits[1]

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ABSTRACT

Nesfatin-1, a product of the nucleobindin 2 (*NUCB2*) gene, purportedly plays important roles in whole-body energy homeostasis. Experiments were conducted to determine how *NUCB2* expression in fat depots may be controlled in the pig and to test the hypothesis that nesfatin-1 regulates appetite and LH secretion in the gilt. Prepubertal gilts were used to study expression of *NUCB2* in fat and the effects of intracerebroventricular (i.c.v.) injection of nesfatin-1 on food intake and pituitary hormone secretion. Growing pigs (gilts and barrows at 22 wk of age, n = 1,145) or sexually mature gilts (n = 439) were used to test association of SNP in the *NUCB2* gene with growth traits. The expression of *NUCB2* was similar for subcutaneous fat compared with perirenal fat. An i.c.v. injection of the melanocortin-4 receptor agonist [Nle^4, d-Phe^7]-α-melanocyte-stimulating hormone did not alter expression of *NUCB2* mRNA in the hypothalamus but reduced ($P = 0.056$) *NUCB2* mRNA expression in subcutaneous fat. Short-term (7 d) submaintenance feeding reduced ($P < 0.05$) BW and did not alter expression of mRNA for *NUCB2*, visfatin, or leptin but increased ($P < 0.05$) expression of adiponectin mRNA in fat. Central injection of nesfatin-1 suppressed ($P < 0.001$) feed intake. Secretion of LH was greater ($P < 0.01$) after i.c.v. injection of nesfatin-1 than after saline. Single nucleotide polymorphisms in the porcine *NUCB2* gene were not associated with adiposity of growing pigs or age at puberty in gilts but were associated ($P < 0.05$) with BW at puberty. These data indicate that *NUCB2* is expressed in fat depots of the pig and that the level of expression is sensitive to stimulation of appetite-regulating pathways in the hypothalamus. It is confirmed herein that nesfatin-1 can regulate appetite in the pig and affect the gonadotropic axis of the prepubertal pig. Association of SNP in the porcine *NUCB2* gene with BW at puberty suggests that regulation of appetite by nesfatin-1 in the pig affects growth, which may have important consequences for adult phenotypes.

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1. Introduction

It is well accepted that adipose tissue is not simply a storage depot for energy but also acts as an endocrine organ by secreting biologically active molecules called adipokines [1,2]. In the pig, adipokines play important roles in regulating economically important traits such as feed intake, tissue growth, and reproduction [3,4]. Thus, identifying new adipokines and defining their biological role in the pig is an important area of research.

Nesfatin-1 (for NEFA/nucleobindin2-encoded satiety- and fat-influencing protein-1) is an 82-amino acid product of the nucleobindin 2 (*NUCB2*) gene and was found to affect feed intake and to reduce BW in rats [5]. Nesfatin-1 is expressed and secreted from subcutaneous (SC) fat explants from humans and mice [6]. In addition, nesfatin-1 expression and secretion are increased on differentiation of 3T3-L1 cells into mature adipoyctes [5,6]. Moreover, *NUCB2* expression was greater in the adipocyte fraction of fat than in the stromal vascular fraction [6], adding further support to the concept of nesfatin-1 as an adipocyte-derived factor. Concentrations of nesfatin-1 in serum are, in general, positively correlated to body mass index in humans [6,7], and nesfatin-1 crosses the blood–brain barrier of the pig via a nonsaturatable mechanism [8,9]. Nesfatin-1 protein is also expressed in areas of the central nervous system (CNS) that are pivotal in regulating energy homeostasis [10]. Expression of nesfatin-1/*NUCB2* in the CNS and SC fat is sensitive to alterations in energy balance [5,6,11,12], suggesting a role for nesfatin-1 in energy homeostasis.

The neuroanatomical distribution of nesfatin-1 neurons in areas of the hypothalamus that integrate energy balance and reproduction (ie, the arcuate nucleus) suggest a possible role of nesfatin-1 in metabolic regulation of reproductive function. Hypothalamic expression of *NUCB2* mRNA increases near the pubertal transition in gonadotropin secretion in rats [13]. Intracerebroventricular (i.c.v.) infusion of *NUCB2* antisense-morpholino oligonucleotides suppressed LH secretion and delayed puberty in female rats [13]. Collectively, these data indicate that nesfatin-1 participates in appetite regulation and energy homeostasis and may have a role in conferring metabolic regulation on the gonadotropic axis. The objective of these studies was to determine how *NUCB2* mRNA in fat depots of the pig may be regulated and to test the hypothesis that nesfatin-1 can regulate feed intake and LH secretion in the pig.

2. Materials and methods

2.1. Animals

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Georgia and the US Meat Animal Research Center and were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching [14]. Pigs used for gene expression and i.c.v. studies were a commercial crossbred composite (sow line C42, boar line 280; Pig Improvement Corp, Franklin, KY, USA). To determine whether there were differences in expression of *NUCB2* mRNA in SC, perirenal, and mesenteric fat depots, 7 gilts that weighed approximately 100 kg and that had undergone ovariectomy at least 30 d earlier were used. To determine the effect of energy balance on expression of *NUCB2* mRNA in SC fat, ovary intact gilts (96.2 ± 2.1 kg) were blocked by BW and randomly assigned to 1 of 2 treatments (n = 8 per treatment): full-fed (200% ME requirement) or feed-restricted (75% ME requirement) for 7 d. Pigs were fed to appetite daily at 7:00 AM and 4:00 PM and had ad libitum access to water. The diet was formulated to meet National Research Council guidelines (NRC 1988) for growing swine. Animals were maintained in individual pens under controlled temperature (22°C) with 12-h light–dark cycles. Pigs used to test for associations of SNP in the porcine *NUCB2* gene with growth and reproductive traits were from a Yorkshire-Landrace-Duroc resource population developed for identification of QTL for production traits [15,16]. Growing pigs (gilts and barrows at 22 wk of age, n = 1,145) or sexually mature gilts (n = 439) were used to test association of SNP in the *NUCB2* gene with growth and weight traits. To test association of SNP in the *NUCB2* gene with age at puberty, 740 gilts were used.

2.2. Predicted nesfatin-1 protein sequencing

The human *NUCB2* sequence (NM_005013.2) was used to search porcine expressed sequence tags (EST; PubMed) that were homologous, and several ESTs were identified. The sequence BQ597948, corresponding to the 3′ end of human *NUCB2* mRNA, was used to design porcine-specific primers and to probe for quantitative real-time reverse-transcription PCR because it was contained on the Affymetrix porcine microarray used in our previous experiment [17]. Porcine EST CK460191, corresponding to the 5′ end of the human *NUCB2* mRNA, was used to deduce the predicted protein sequence for porcine nesfatin-1 (Fig. 1).

2.3. Effects of i.c.v. injection of α-melanocyte-stimulating hormone

Expression of *NUCB2* mRNA was measured in SC fat available from a previously published study [17] that evaluated the effects of i.c.v. injection of the melanocortin-4 receptor agonist [Nle^4, d-Phe^7]-α-melanocyte-stimulating hormone (NDP-MSH) on gene expression in fat, liver, and hypothalamic tissue. The description here of i.c.v. injections and tissues collected is abridged for brevity. Eighteen pigs (n = 12 gilts and 6 castrate males) weighing 64 ± 2 kg were surgically fitted with i.c.v. cannula in the right lateral ventricle with the use of a stereotaxic procedure described previously [18,19]. Placement of the i.c.v. cannula was verified for each animal by x-ray. One week later, feeders were removed at 7:00 AM, and i.c.v. injections of 150 μL of 0.9% saline (n = 9) or 10 μg of NDP-MSH (Bachem, Torrance, CA, USA) in 150 μL of 0.9% saline (n = 9) were administered at 8:00 AM. Feeders were placed in pens, and cumulative food intake was determined at 12 and 24 h after feed presentation. All pigs were harvested 24 h after i.c.v. injection, and hypothalamus, liver, and 10th rib layer of SC fat were collected to determine mRNA expression. The entire hypothalamus was collected by making the

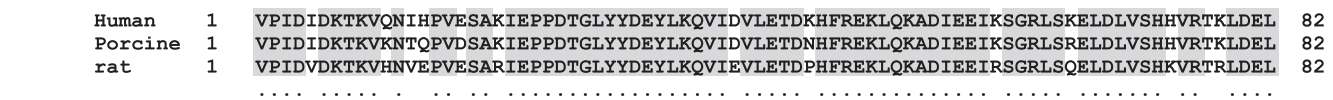

Fig. 1. Alignment of human and rat nesfatin-1 with the predicted porcine nesfatin-1 protein sequence. Shading represents amino acids that are conserved among the 3 species.

following cuts: rostral to the optic chiasm, rostral to the mammillary body, lateral to the hypothalamic sulci, and ventral to the anterior commissure. Effects on feed intake and global changes in gene expression are reported elsewhere [17].

2.4. Effects of i.c.v. injection of nesfatin-1

Twelve ovary intact prepubertal gilts (87.9 ± 2.1 kg) were surgically fitted with i.c.v. cannula as described above. At least 1 wk after the placement of i.c.v. cannula, and 24 h before treatment, all gilts were fitted with an indwelling jugular catheter (micro-Renathane tubing, 0.04 mm ID × 0.08 mm OD × 100 cm long; Braintree Scientific Inc, Braintree, MA, USA). Animals were randomly assigned to treatment (n = 4 per treatment): saline (150 μL of 0.9% saline), leptin (6.25 pMol, 100 μg of recombinant human leptin; R&D Systems, Minneapolis, MN, USA), or nesfatin-1 (10.5 nMol, 100 μg of human nesfatin-1; Phoenix Pharmaceutical, Burlingame, CA, USA) in 150 μL of 0.9% saline. Serial blood samples were drawn every 15 min for 3 h before and 4 h after i.c.v. treatment. Concentrations of LH in serum were quantified by RIA [20]. The reference standard for LH (AFP-10506A) was provided by Dr A.F. Parlow, Scientific Director of the National Institutes of Health, National Institute of Diabetes, Digestive, and Kidney Disease, National Hormone and Peptide Program. Sensitivity of the assay was 0.15 ng/mL. Intra-assay and inter-assay CVs were 7.8% and 12.9%, respectively. Concentrations of LH were subjected to ANOVA with repeated measures by using the MIXED procedure of SAS (SAS Institute Inc, Cary, NC, USA). The model included treatment, time, and the treatment-by-time interaction, with a compound symmetric function used to model the covariance structure for the repeated measures. The Kenward–Roger estimate was used for the denominator degrees of freedom.

On the day of the experiment, feeders were removed from the pens at 8:00 AM after the pigs had consumed their morning meal (pigs fed to appetite). After the last blood sample was collected (4 h after i.c.v. treatment), feeders were placed in all pens, and food intake was monitored at 4, 20, 44, and 68 h after feed presentation (corresponding to 8, 24, 48, and 72 h after i.c.v. treatment). Data were subjected to a mixed model ANOVA with repeated measures as described above.

2.5. Tissue collection and isolation of total RNA

Tissues were snap-frozen in liquid nitrogen and stored at −80°C until isolation of RNA. Total RNA was isolated by extraction with tri-reagent (QIAzol Lysis Reagent; Qiagen, Valencia, CA, USA), followed by isopropanol precipitation. After resuspending the pellet in water, RNA was then purified by passing it over purification columns (RNEasy midi kit; Qiagen). Quantity and quality of RNA were determined by microfluidic analysis with an Experion automated electrophoresis system (Bio-Rad, Hercules, CA, USA).

Table 1
Primer pairs, reporter sequence, and GenBank accession number for each gene.

Gene	Symbol	GenBank accession number	Sequence (5′ to 3′)	Design strand	Amplicon length, bp
Angiopoietin-like 4	*ANGPTL4*	NM 001038644	Forward primer: ACTGCCAAGAGCTGTTTGAAGA Reverse primer: CCATCTGAGGTCATCTTGCAGTTAA Reporter: CTTTGCCGCTCTCCC	Reverse	106
Adiponectin	*ADIPOQ*	NM 214370	Forward primer: ACTAGCCCTGCCCAGTCT Reverse primer: TGGGATACCCGCCATCCA Reporter: ACTACTGCCCATGCCC	Forward	100
Fatty acid synthase	*FASN*	NM 001099930	Forward primer: GCTTGTCCTGGGAAGAGTGTAAG Reverse primer: CACCGTGTCTTTGGAGTTGTG Reporter: CAGCGCTGCCCCC	Forward	77
Leptin	*LEP*	NM 213840	Forward primer: GACGATTGTCACCAGGATCAGT Reverse primer: CGGTGACCCTCTGTTTGGA Reporter: CACATGCAGTCTGTCTCC	Forward	68
Nesfatin	*NUCB2*	BQ597948	Forward primer: GTGGACTTCAAGCTTCAAATGTGT Reverse primer: GTGGACTTCAAGCTTCAAATGTGT Reporter: CTGCTGCACAACCTGATGAT	Forward	140
Visfatin[a]	*NAMPT*	NM 001031793.2	AGGAGACCTTGAGGAATACGGTCAT[b]	NA	98
18S ribosomal RNA	*18S*	NR 002170	Forward primer: AGGGCATCACAGACCTGTTATTG Reverse primer: CCCCAACTTCTTAGAGGGACAAG Reporter: CAGCCACCCGAGATTG	Reverse	71

Abbreviation: NA, not applicable.
[a] Noncustom Taqman gene expression assay ID Ss03379479_u1; target exon 14.
[b] Context sequence of noncustom Taqman gene expression assay.

2.6. Quantitative real-time reverse transcription PCR

Custom Taqman gene expression assays for angiopoietin-like 4 (*ANGPTL4*), fatty acid synthase (*FASN*), adiponectin, leptin, visfatin, and nesfatin-1 (Table 1) were designed by Applied Biosystems (Foster City, CA, USA). These were chosen because they are markers of fatty acid storage and respond differentially to alterations in energy balance. The porcine 18S rRNA was used as the endogenous control. Three micrograms of total RNA were transcribed into cDNA in a volume of 20 μL with Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) by using random hexamers. The cDNA was diluted 1:10 for use as template in triplicate amplification reactions, which consisted of 1 μL of diluted cDNA, 10 μL of iQ Supermix (Bio-Rad), 0.8 μL of 20× custom TaqMan gene expression assay, and water to a total volume of 20 μL. To determine the reaction efficiency of each assay probe, a series of reactions were performed by using a template pool of all available cDNA in serial dilution (3- to 2,187-fold serial dilutions). Reaction efficiency ranged from 0.8 to 1.0. The PCR reactions were performed with a CFX 96 Real-Time PCR Detection System (Bio-Rad). Thermal cycling parameters were 1 cycle of 95°C for 10 min, followed by 40 cycles of denaturing (95°C for 15 s) and annealing/extension (60°C for 60 s). Cycle threshold (Ct) values of triplicate reactions were compared, and reactions that did not fall within 1 cycle of each other were repeated. The Ct values of within-sample triplicate wells were averaged, and relative differences in gene expression were calculated by the Relative Expression Software Tool [21] and by the comparative Ct ($2^{-\Delta\Delta Ct}$) method [22] by using the MIXED procedure of SAS (SAS Institute Inc) with fat depot or treatment as fixed effects and pig as a random effect.

2.7. SNP discovery and association of NUCB2 SNP with growth and reproductive traits

Primer pairs for amplification of *NUCB2* from genomic DNA were designed from porcine sequences deposited in GenBank by using Primer 3 [23] (http://frodo.wi.mit.edu/primer3/input.html). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Amplification and sequencing were performed in a Dyad PTC-0220 DNA engine (Bio-Rad) by using 0.5 U of Hot Star *Taq* polymerase (Qiagen), 1× supplied buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.8 μM of each primer, and 100 ng of genomic DNA of 12 breeding boars from the US Meat Animal Research Center population in 25-μL reactions. Sequencing reactions were prepared as described [16] and were sequenced on an ABI 3730 capillary sequencer (Applied Biosystems). Polymorphisms were identified with Polyphred and assessed with Consed (http://www.phrap.org).

Multiplex assays for use in the Sequenom MASSARRAY system were designed with MASSARRAY Assay Design software (Sequenom, San Diego, CA, USA). Reaction conditions were performed as suggested by Sequenom iPLEX chemistry. Genotypic data were analyzed with the QTL Association option of Mendel version 12.0 [24], which uses full pedigrees. The statistical model included breeding season (n = 4) and age as covariates and line as a fixed effect.

3. Results

3.1. Expression of NUCB2 in porcine adipose tissue

A human *NUCB2* mRNA sequence was blasted against porcine EST to identify the pig homolog. The porcine EST CK460191 corresponded to the 5′ end of the human *NUCB2* mRNA and contained the putative ATG start site of the porcine gene. This porcine sequence coded for an 82-amino acid protein that shared 92% and 85% sequence identity to human and rat nesfatin-1, respectively (Fig. 1). Expression of *NUCB2* in the hypothalamus and liver did not differ with NDP-MSH treatment (Table 2). Pigs that received an i.c.v. injection of NDP-MSH tended ($P = 0.056$) to have less abundant expression of *NUCB2* mRNA in SC fat than pigs receiving i.c.v. injections of saline (Table 2).

Fat depot-dependent expression of mRNA for adiponectin, leptin, visfatin, and *NUCB2* was observed. Adiponectin mRNA was greater ($P < 0.05$) in perirenal fat than in mesenteric fat (Fig. 2). Expression of adiponectin in SC fat was intermediate. More abundant ($P < 0.05$) expression of leptin mRNA was observed in SC fat than in mesenteric or perirenal fat, which were not different from each other (Fig. 2). Expression of visfatin and *NUCB2* mRNA was greatest ($P < 0.05$) in mesenteric fat compared with perirenal or SC fat (Fig. 2). Differences in gene expression were observed in fat depots that correlated with loss of BW (Fig. 3) because of negative energy balance induced with feed restriction. Abundance of mRNA for *ANGPTL4* was greater ($P < 0.05$) and *FASN* was less ($P < 0.001$) in SC fat of feed-restricted pigs than in full-fed control pigs (Fig. 4). Feed-restricted pigs had greater ($P < 0.01$) expression of adiponectin than full-fed control pigs, but expression of leptin, visfatin, or *NUCB2* mRNA did not differ with amount of feed intake (Fig. 4).

3.2. Effects of i.c.v. injection of nesfatin-1

Central injection of leptin suppressed ($P < 0.001$) cumulative feed intake compared with saline-treated control pigs (Fig. 5). Similarly, animals receiving i.c.v. injection of nesfatin-1 ate less feed than saline-treated pigs (Fig. 5). At 24 h after i.c.v. injection, leptin- and nesfatin-1–treated pigs began to eat at a similar rate compared with

Table 2
Expression ratios of *NUCB2* mRNA in the hypothalamus, liver, and MSQ layer of backfat in pigs 24 h after intracerebroventricular injection of saline or 10 μg of the melanocortin 4 receptor agonist NDP-MSH.

Tissue	Expression ratio[a]	*P* value[b]
Hypothalamus	−1.059	0.6623
Liver	−1.051	0.6866
MSQ backfat	−1.452	0.0560

Abbreviations: MSQ, middle subcutaneous; NDP-MSA, [Nle^4, D-Phe^7]-α-melanocyte-stimulating hormone (Bachem, Torrance, CA, USA); *NUCB2*, nucleobindin 2.

[a] Log_2 ratios are a comparison of quantitative real-time PCR expression values from saline versus NDP-MSH–treated pigs. A negative value is downregulated with NDP-MSH treatment.

[b] Determined by pairwise fixed reallocation randomization test within the Relative Expression Software Tool.

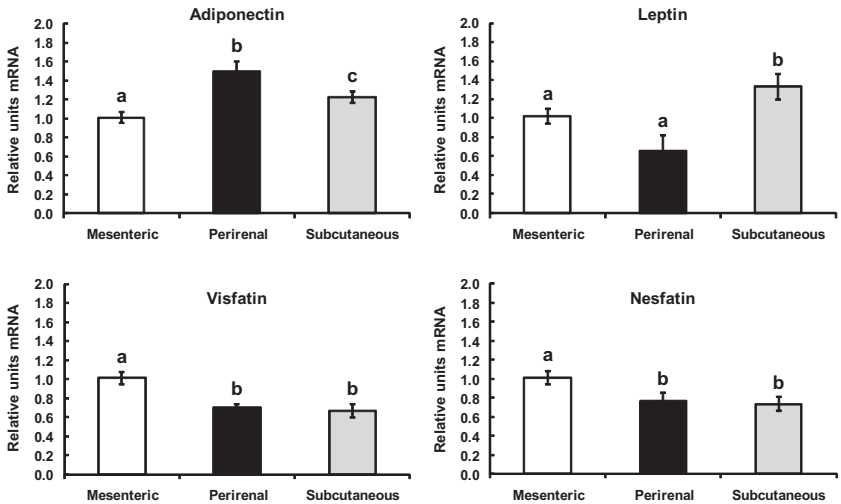

Fig. 2. Relative expression of mRNA in mesenteric (open), perirenal (black), and subcutaneous (shaded) fat depots of gilts that had undergone ovariectomy. [a,b,c] $P < 0.05$.

saline-injected control animals, but there was no compensation in food intake. Concentrations of LH were not analyzed for leptin-treated pigs because 2 of the 4 catheters failed to remain patent. Secretion of LH in nesfatin-1– and saline-treated pigs are depicted in Figure 6. A treatment-by-time interaction ($P < 0.01$) was observed for mean concentrations of LH. Mean concentrations of LH were not different ($P = 0.71$) for control- and nesfatin-1–treated pigs during the 2 h before i.c.v. injection; however, LH secretion was increased ($P < 0.001$) after i.c.v. injection of nesfatin-1 and was greater ($P < 0.0001$) than for pigs receiving i.c.v. saline (Fig. 6).

3.3. *NUCB2* SNP and association with growth and reproductive traits

A total of 7 SNPs were identified in porcine *NUCB2* (GenBank dbSTS accession numbers BV728450, BV72845, and BV728452; dbSNP ss numbers 95215024 to 95215030). The dbSNP reference SNP numbers for 57807_199, 57813_217, and 57813_91 are rs81219683, rs81215763, and rs81219689, respectively. No association was observed of backfat at the first, last, or lumbar areas measured in pigs at 22 wk of age with SNP in the porcine *NUCB2* gene (Table 3). One SNP in the porcine *NUCB2* gene was associated ($P < 0.05$) with BW at 22 wk of age and with ADG from 8 to 22 wk of age (Table 3). Age at puberty, as determine by age at first estrus, was not associated with SNP in the *NUCB2* gene, but BW at puberty was associated (Table 4).

4. Discussion

Results from in silica analysis indicated that porcine nesfatin-1 protein had greater sequence identity with human than rat nesfatin-1; thus, human nesfatin-1 was chosen to test its i.c.v. effects in pigs. Central injection of pigs with nesfatin-1 suppressed feed intake, in agreement with results from rats [5,25] and mice [26]. This indicates that nesfatin-1 may be a key regulator in the central control of appetite in the pig. Leptin and nesfatin-1[5] both act to influence food intake through central melanocortin receptors, which are important for regulating appetite, growth, and adiposity in swine [27]. The effects of nesfatin-1 on appetite, however, appear to be independent of the action of leptin [28]. Nesfatin-1 has a potent anorectic effect in animals that are resistant to the effects of leptin [5,29], and i.c.v. injection of nesfatin-1 antibodies did not block the inhibitory effect of leptin on food intake in the rat [5].

Nesfatin protein is expressed in areas of the pig brain involved in sensing energy balance and regulating food intake; chiefly the paraventricular nucleus (PVN), lateral hypothalamic area, arcuate nucleus, and supraoptic nucleus of the hypothalamus and the area postrema of the nucleus tractus solitarius, and the dorsal motor nucleus of the vagus in the brainstem [10]. Expression of *NUCB2* mRNA in the hypothalamus of rats is reduced with fasting [5,13], and refeeding activated cFos expression in nesfatin-1 cell bodies of the PVN [11]. Moreover, shifting concentrations of glucose in plasma of rats activated nesfatin-1 cell bodies in

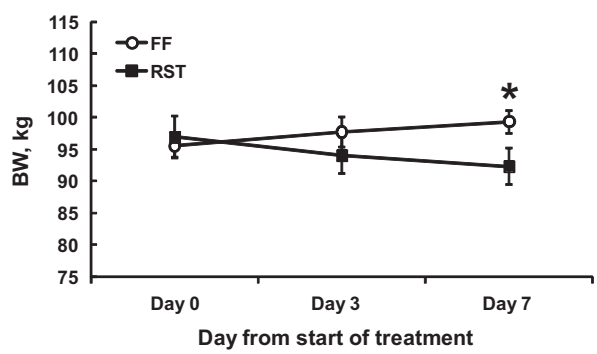

Fig. 3. Change in body weight of gilts that were full-fed (FF) or feed restricted (RST) for 7 d. * $P < 0.05$.

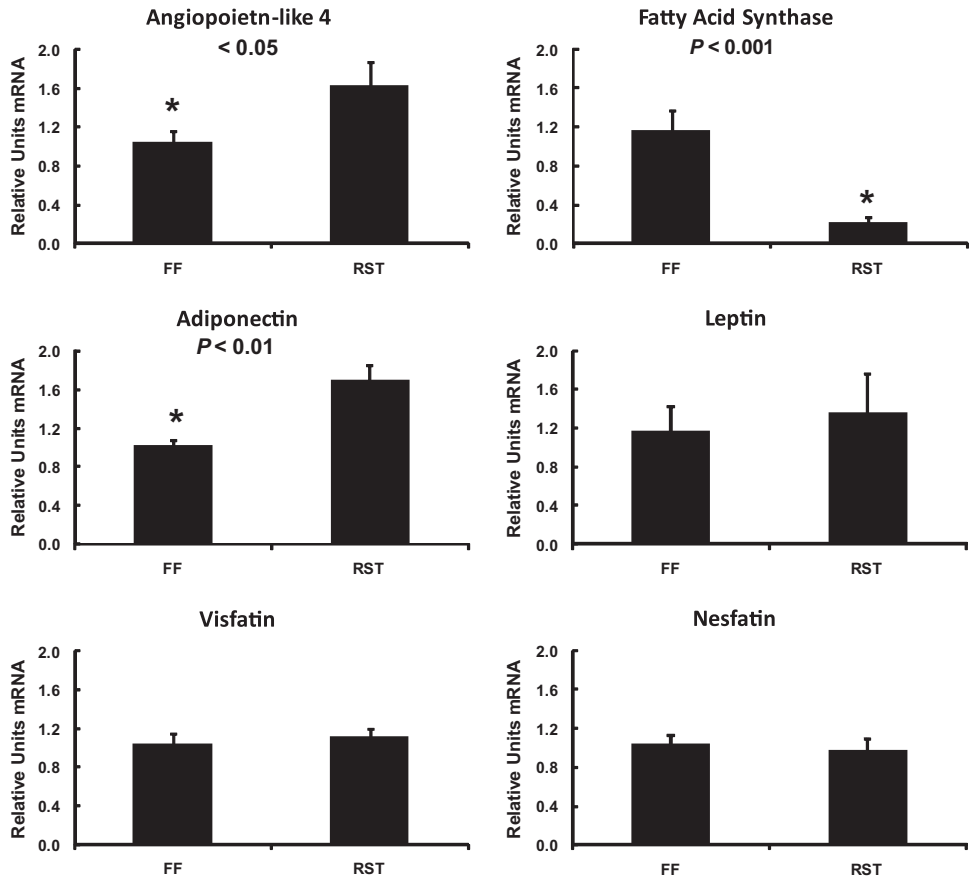

Fig 4. Relative expression of mRNA in subcutaneous fat of gilts that were full-fed (FF; n = 8) or feed restricted (RST; n = 8) for 7 d. Full-fed was set to 1.0 to determine fold change. * FF is different from RST.

the hypothalamus as well as the nucleus tractus solitarius and dorsal motor nucleus of the vagus [12], suggesting that activation of nesfatinergic neurons throughout the CNS is important in sensing metabolic status and altering food intake to control energy homeostasis. Gaigé et al [10] reported that feeding the mycotoxin deoxynivalenol to weanling pigs activated nesfatin cell bodies in the hypothalamus and brainstem. They speculated that nesfatin neurons in the CNS are involved in the suppression of appetite in pigs that consume feed with high levels of mycotoxins. Of course mycotoxicosis is multifaceted, but the present results confirm that nesfatin-1 can suppress appetite in the pig. The anorexigenic actions of nesfatin-1 involve oxytocin and corticotrophin-releasing hormone–expressing cells in the PVN [25,28].

Concentrations of LH in serum of prepubertal gilts were greater after treatment with nesfatin-1 than after saline. When prepubertal female rats were given i.c.v. injections of nesfatin-1, LH secretion was increased only 2-to 3-fold; however, when rats were fasted for 48 h, nesfatin-1 stimulated a 9-fold increase in LH [13]. The greater LH response to nesfatin-1 in fasted rats may be attributed to reduced concentrations of nesfatin-1 entering the brain from the blood, or a decrease in central expression of *NUCB2* and paracrine action of nesfatin-1 in the hypothalamus. Expression of *NUCB2* in the PVN of the hypothalamus and in SC fat of rats is suppressed with fasting [5,13]. In the present study, gilts were fed to maintain commercially acceptable growth rates and were allowed to consume the morning meal on the day of treatment, which may have limited the LH secretory response.

We found no association of SNP in the porcine *NUCB2* gene with adiposity traits of pigs (barrows and gilts) at 22 wk of age. Zegers et al [30] reported that SNPs in the *NUCB2* gene were associated with the amount of body fat in adult humans. Interestingly, this was observed for men but not for women. There was an association of porcine *NUCB2* gene with BW and BW gain at 22 wk of age as well as BW of gilts at puberty. These associations should be confirmed in additional animals, but the fact that nesfatin-1 controls appetite in the pig strengthens the probability that these SNP associations are in fact real. Because of the positive relationship of BW and body fatness with age at puberty in the gilt and the effect of nesfatin-1 to stimulate LH, the potential for associations of age at first estrus with genetic variation in *NUCB2* was tested; however, no association was found. This may result because thresholds in BW gain and adiposity of gilts are necessary for puberty to occur at younger ages [31] but are not sufficient, in and of themselves, to initiate reproductive cycles.

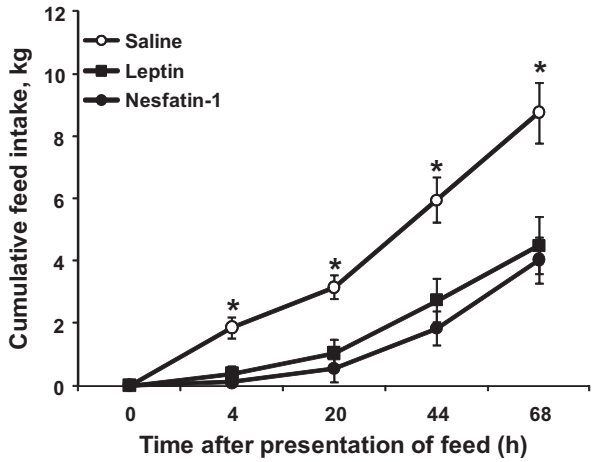

Fig. 5. Effects of intracerebroventricular injection of leptin and nesfatin-1 on cumulative feed intake at 4, 20, 44, and 68 h after feed presentation (corresponds to 8, 24, 48, and 72 h after intracerebroventricular treatment). A treatment-by-time interaction ($P < 0.001$) was observed. * Different from saline-injected control animals ($P < 0.01$).

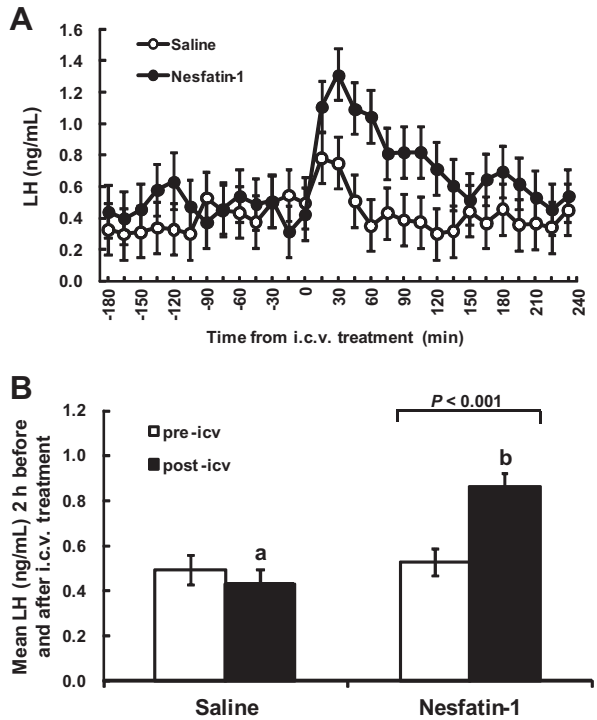

Fig. 6. Concentrations of LH in plasma (A) of gilts receiving intracerebroventricular (i.c.v.) injection of saline or nesfatin-1 (n = 4 per treatment) and mean concentrations of LH (B) during the 2 h before and 2 h after i.c.v. injection (time 0). Least squares means are presented. After i.c.v. treatment, LH for nesfatin-1–treated animals was greater than for saline-treated animals. [a,b] $P < 0.0001$.

Because nesfatin-1 was identified as an adipose-secreted factor involved in energy balance, we sought to examine how *NUCB2* expression in fat depots of the pig might be regulated. Expression of *NUCB2* was not different in SC compared with perirenal adipose tissue depots, but it was somewhat greater in mesenteric adipose tissue. In contrast, expression of *NUCB2* mRNA and nesfatin-1 protein was reported to be greater in SC adipose tissue than in omental adipose tissue of both mice and humans [6]. Nutritional restriction was used to induce negative energy balance, resulting in significant differences in BW after 7 d, and was supported by expected differences in gene expression of adipose tissue. Downregulation of FASN in feed-restricted gilts reflects a reduction in lipid biosynthesis, whereas upregulation of ANGPTL4 further antagonizes lipogenesis by inhibiting lipoprotein lipase activity [32]. Caloric restriction increases tissue expression and serum concentrations of adiponectin [33–35] and is consistent with expression of adiponectin in the present study. The failure of nutrient restriction to alter gene expression for leptin was not unexpected and is consistent with the previous report indicating that expression of leptin mRNA in adipose tissue of pigs is reduced with fasting but not submaintenance feeding [36]. Likewise, neither visfatin nor *NUCB2* mRNA expression in adipose tissue was affected by nutritional restriction. Ramanjaneya et al [6] found that fasting reduced nesfatin-1/*NUCB2* expression in SC adipose tissue of mice. Like leptin, complete food deprivation may be required to alter *NUCB2* expression in adipose tissue of pigs. Regardless, these data indicate that pig adipose tissue expresses *NUCB2* mRNA. It should be noted that changes in mRNA do not always correspond to differences in protein expression

Table 3
Association of SNPs in the porcine nucleobindin 2 gene with BF, BW, and ADG in commercial type white composite pigs (n = 1,145).

Marker	Chromosome	Position[a]	Positive allele	Negative allele	Estimated effect				
					BF[b] first rib	BF last rib	BF lumbar	BW[c]	ADG[d]
57807_199	2	39132035	A	G	0.1440	0.1462	0.1620	0.2779	3.6×10^{-3}
57813_217	2	39118044	C	T	0.0789	0.0546	0.0192	0.5317	4.2×10^{-3}
57813_91	2	39118070	T	C	0.0229	0.0190	0.0092	0.3470[e]	5.4×10^{-3}[e]

Abbreviations: ADG, average daily gain; BF, back fat.
[a] Position of SNP on chromosome 2 in *Sus scrofa* build 9.2.
[b] Backfat at the first rib, last rib, and lumbar area determined by ultrasound scanning at 22 wk of age (mean ± SD = 16.3 ± 0.2, 12.4 ± 0.2, and 13.7 ± 0.2 mm, respectively).
[c] Body weight at 22 wk of age (mean ± SD = 97.1 ± 0.4 kg).
[d] Average daily gain from 8 to 22 wk of age (mean ± SD = 0.79 ± 0.01 kg/d).
[e] $P < 0.05$.

Table 4
Association of SNPs in the porcine nucleobindin 2 gene with age (n = 740) and BW (n = 439) at puberty in Yorkshire-Landrace-Duroc gilts.

Trait[a]	Marker	Positive allele	Negative allele	Estimated effect	*P* value
Pubertal age (d)	57807_199	A	G	0.2857	0.67
	57813_217	C	T	0.1347	0.79
	57813_91	C	T	0.1696	0.70
Pubertal BW (kg)	57807_199	A	G	0.3062	0.73
	57813_217	C	T	1.7441	0.01
	57813_91	T	C	1.4070	0.02

[a] Mean ± SD pubertal age = 196.7 ± 0.6 d; mean ± SD pubertal BW = 128.4 ± 0.9 kg.

and that the role of nesfatin-1 in adipose tissue biology is poorly understood.

Central injection of pigs with NDP-MSH suppressed food intake for 24 h and caused the differential expression of 278 genes in the hypothalamus, 249 genes in the liver, and 5,066 genes in SC fat tissue [17]. The dynamic changes in gene expression in fat of NDP-MSH–treated pigs were associated with anabolic and catabolic processes similar to the transcriptional events of metabolic adaptation to food deprivation. These included downregulation of *FASN*, lipoprotein lipase, and leptin genes and upregulation of *ANGPTL4* and uncoupling protein 3 genes [17]. A tendency was observed for NDP-MSH–treated pigs to have less abundance of *NUCB2* mRNA in SC adipose tissue than saline-treated pigs. Cumulatively, the results reported herein for *NUCB2* mRNA abundance suggests that regulation of nesfatin-1 expression in adipose tissue of pigs may be similar to that of leptin. No differences were observed in expression of *NUCB2* mRNA in the hypothalamus of pigs that received i.c.v. injections of either saline or NDP-MSH. Central injection of rats with α-MSH stimulated *NUCB2* expression, but only in the PVN of the hypothalamus [5]. The approach taken here used RNA from the whole hypothalamus, and differences in localized expression of *NUCB2* mRNA in response to NDP-MSH could not be determined for these pigs. After NDP-MSH treatment, hypothalamic expression of neuropeptide-Y was increased and promelanin-concentrating hormone decreased with no differences in expression of agouti-related protein and pro-opiomelanocortin [17], clearly indicating that in these pigs NDP-MSH treatment differentially affected neurologic pathways that regulate appetite.

In summary, central injection of nesfatin-1 suppressed appetite and stimulated LH secretion in prepubertal gilts. These data confirm that nesatin-1 can regulate food intake in the pig and can act in the hypothalamus to affect the gonadotropic axis of prepubertal animals. Expression of *NUCB2* mRNA in SC adipose tissue of the pig did not change with short-term feed restriction, but stimulation of MSH-sensitive pathways in the hypothalamus reduced *NUCB2* mRNA expression in fat. How expression and secretion of nesfatin-1 from adipose tissue contributes to adipose tissue biology and whole-body metabolism remains poorly understood. Genetic variation in the porcine *NUCB2* gene was not associated with measure of body fat in young growing pigs but was associated with BW at puberty, which could have important implications in adult animals.

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